REMARKS

The Present Invention

The present invention is directed to unexpected improvements in the set-up, performance, and results of multiplex PCR reactions involving the coamplification of two or more target nucleic acids having different sequence compositions. Included among the improvements is the elimination of the need for primer optimization, i.e, the determination of the optimal molar concentration of each primer based on its target, in a multiplex PCR reaction, which is labor intensive and time-consuming. By following the method of the present invention, an equimolar concentration of all primers can be added to a multiplex PCR reaction regardless of the size or number of targets to be amplified in the reaction.

The improvement is directed to the use of a chemically-modified hotstart DNA polymerase, such as HotStarTaq® (Qiagen GmbH, Germany), in combination with a polymeric volume exclusion agent in multiplex PCR reactions. It has surprisingly been discovered that, even with multiple target nucleic acids of varying lengths and comparable copy number present in a multiplex PCR reaction mixture, in the presence of a hotstart DNA polymerase and a volume exclusion agent, equimolar concentrations of all primers may be used in the reaction and all targets are amplified to strong levels, as demonstrated in Applicants' working examples.

As described in the present specification, prior to Applicants' discovery, attempts to optimize product yield in multiplex PCR reactions, for example, by adjusting the annealing temperature to preferentially hybridize a particular primer or primers, or by adjusting the concentration of primers ("primer-biasing"), often solved one problem, but created another, e.g., it was found that when adjusting the temperature of the reaction to favor certain primer hybridization, the melting temperature between different primers had to be relatively large to optimize particular binding by temperature adjustment, and adjusting primer concentration to optimize amplification has met with limited success. (See, Applicants' specification at page 5, lines 10-25.) As such, it was surprising that, in a multiplex PCR reaction, the inclusion of a chemically-modified hotstart DNA polymerase together with a volume exclusion agent in a standard PCR reaction with equimolar concentrations of all primers (thereby eliminating a labor-intensive step from the set up of the reaction) led to good yields of all amplified target nucleic acids and eliminated the need for other "specialized" adjustments in reaction parameters. As disclosed in the specification:

"Some of the [problems of the prior art] described above can be eliminated by the use of a PCR hot start technique described above. However, in co-amplification reactions (multiplex reactions) using equimolar concentrations of all primers it is frequently observed that the yield of some amplification products is much lower than from others. **Some amplification products may even not be detectable at all, even when a comparable number of copies of the target DNA sequence are present within the reaction.** This may be even more pronounced when low concentrations of primers are used, which is required to minimize the influence of the reactions on each other. Strong differences in product yield are due to differences in hybridization of the primers to their respective target sequence and extension of such annealed primers. When such differences in product yield are observed, **researchers typically need to adapt the concentration of primers to obtain comparable product yield. Establishment of the optimal molar ratio of primers is typically very difficult to perform.**"

* * *

"The hybridization of primers in each cycle of PCR can be increased by a dramatically prolonged annealing step in each PCR cycle. This dramatically increases the overall time for an multiplex PCR assay, which decreases convenience and is unwanted or not acceptable in most cases."

* * *

"The problems noted above are overcome with methods which significantly enhance the performance of multiplex PCR reactions. The present invention provides methods and reagents for enhanced performance of multiplex PCR reactions. Such efficient conditions for multiplex PCR amplification [involve] the use of a hot-start DNA polymerase within the PCR reaction **in combination with** a volume excluding reagent. The positive effect on multiplex PCR is due on one hand to the use of a hot-start enzyme, which maximizes PCR efficiency by minimizing unwanted non-specific hybridization and extension of primers or formation of primer-dimers. On the other hand, the presence of a volume excluding reagent enhances the hybridization rate of all primers within the reaction. The presence of a volume excluding reagent in the reaction results in significantly increased product yield of targets that exhibit poor amplification under multiplex PCR conditions (limited primer concentrations) in the absence of the volume excluding reagent.

* * *

"Surprisingly, it has been found that it is possible to perform multiplex PCR using even equimolar concentrations of all primers. According to the invention product yield from targets that exhibit poor amplification can be increased to acceptable levels by the use of a volume excluding reagent in combination with a hot-start DNA polymerase." (Applicants' specification at page 7, line 1, to page 8, line 7.)

As demonstrated in the specification, a hotstart DNA polymerase which has been chemically-reversibly inactivated is particularly advantageous in a multiplex PCR reaction when used in combination with a volume exclusion agent over a standard thermostable DNA polymerase, such as unmodified *Taq* DNA polymerase:

Examples 1 and 2 of the specification, beginning on page 26, describe the coamplification of multiple target nucleic acids in a multiplex PCR reaction (i.e., the PKC, SLP-65, ILGFMAR, c-fos, N-ras, fas, CD19 and CD5 murine genomic loci, all of which vary in size from 192 bp to 1953 bp and vary in sequence) using equimolar concentrations of all primers specific for each loci. Being genomic loci, the copy numbers of these targets were comparable. Amplification reactions were carried out with either a standard thermostable *Taq* DNA polymerase or a hotstart DNA polymerase (HotStarTaq®). The reactions included either 0%, 1%, 3%, or 6% of either dextran or polyethylene glycol (PEG) as volume exclusion agents.

As seen in Figure 1 (dextran as volume exclusion agent), in a multiplex PCR reaction, the combination of hotstart enzyme and dextran (lanes 3-8) shows significant improvement of the yield of <u>all</u> products in the reaction over use of enzyme alone (lanes 1 and 2), AND shows significant improvement over the same multiplex PCR reaction performed with a standard *Taq* polymerase and dextran (lanes 9-16). In the multiplex PCR reaction, standard *Taq* polymerase with or without dextran yielded virtually no PCR except for the c-fos target. Similar results are seen in Figure 2, in which a hotstart DNA polymerase in combination with PEG 8000 is compared with a standard DNA polymerase (*Taq*) and PEG 8000. As seen in Table 3 (page 30) and Fig. 2 of the application, the combination of a hotstart enzyme and PEG volume exclusion agent led to a significant increase in the signal of all PCR target nucleic acids, whereas the use of standard DNA polymerase and PEG led to amplification of only one of the multiplex targets. Therefore, the present invention clearly demonstrates that a multiplex PCR coamplification reaction to amplify multiple target nucleic acids and using multiple sets of primers specific for each target, shows a significant improvement in yield of multiple products when the

amplification reaction is performed in the presence of a hotstart DNA polymerase in combination with a volume exclusion agent.

I. Specification

The Examiner has objected to the incorporation by reference of essential material, in particular the reference of the chemically modified DNA polymerase (HotStarTaq®) on page 27 of the application as disclosed in European Pat. Appln. No. 99 110 426, which corresponds to U.S. Pat. No. 6,183,998.

In response, Applicants have amended the specification at page 27 to delete the reference to a foreign publication and replace it with the description of hotstart DNA polymerases as set forth in U.S. Pat. No. 6,183,998 that was intended to be incorporated. Support for the amendment may be found in U.S. 6,183,998 at col. 3, line 5-21 and 56-59, and col. 12, lines 20-21.

The amendment to the specification is a description of material previously incorporated by reference and no new matter is added by this amendment.

Entry of the amendment to the specification is respectfully requested.

II. 35 U.S.C. §103(a)

The Examiner has rejected Claims 1-2, 4-11, 16, 23, and 24 under 35 U.S.C. §103 as being unpatentable over Backus et al., U.S. Pat. No. 5,705,366[1], in view of Bustin, S.A., *Journal of Molecular Endocrinology*, 25: 169-193 (2000) and further in view of Birch et al., U.S. Pat. No. 5,773,258 ("Birch et al.").

According to the Examiner, with respect to Backus,

> "Backus discloses a method of amplification of multiple target nucleic acids in the presence of a nonionic, polymeric volume exclusion agent." (See, Office Action, page 3.)

With respect to Bustin, the Examiner states,

> "Bustin teaches an overview of the quantitation of mRNA using a variety of methods, including quantitative real-time RT-PCR, a method which incorporates a variety of means of detection, including hybridization probes." (See, Office Action, page 9.)

[1] Applicants note that the Examiner cites U.S. Pat. No. 5,703,366 throughout the Office Action. However, U.S. Pat. No. 5,703,366 is issued to Sting et al. and is entitled "Optical Sensing With Crystal Assembly Sensing Tip".

And the Examiner cites the following passage from the Bustin reference,

> "The accepted method for minimizing these errors and correcting for sample-to-sample variation is to amplify, simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values can be normalized. The ideal internal standard should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by the experimental treatment. In addition, an exogenous control should also be expressed at roughly the same level as the RNA under study." (See, Office Action, page 10.)

The Backus reference does not teach the combination of a volume exclusion agent and a chemically-modified hotstart DNA polymerase for the coamplification of two or more target nucleic acids. In fact, Backus teaches that, although volume exclusion agents are known to increase hybridization rates, they should only be used in PCR in a very limited set of circumstances, e.g., slow hybridization rates or where the nucleic acid is rate limiting in the reaction, because volume exclusion agents lead to high background and an increase in the viscosity of the reaction, making it difficult to handle. (See, Backus, column 2, lines 48-59.)

Backus only allows that the use of a volume exclusion agent in combination with an antibody-inactivated polymerase may be suitable under very limited hybridization conditions, e.g., where there is approximately **100,000-fold** difference in the levels of target nucleic acids, i.e., of high-copy number DNA and low-copy number DNA in the same reaction. Examples 1-4 of Backus disclose a PCR reaction with a DNA polymerase inactivated by the TP4 monoclonal antibody wherein the reaction is comprised of one population of low copy nucleic acid (10 copies of proviral HIV I DNA) and one population of high copy nucleic acid (1,000,000 copies of human β-globin DNA) amplified in the presence of 10% PEG. As seen in Table I of Backus, the best level of PCR amplification in the presence of 10% PEG 8000 only resulted in a 3.4-fold increase over control levels.

In contrast, the present application demonstrates an improvement in a multiplex PCR reaction whereby multiple DNA targets can be co-amplified in high yield without resort to complicated manipulations of reaction time, hybridization conditions, or primer amounts. The key is the combined use of a hotstart DNA polymerase and a volume exclusion agent. The data presented in Applicants' examples demonstrate that use of this combination leads to amplification of multiple targets instead of differential amplification, even where the copy

numbers of the targets are comparable and even where the primers used are added in equimolar concentrations.

The Bustin reference is a review article describing methods for quantifying mRNA gene transcripts by reverse transcription polymerase chain reaction (RT-PCR). According to Bustin, RT-PCR-specific errors in the quantification of mRNA transcripts are compounded by variation in the amount of starting material, particularly when samples are taken from different individuals. The section of Bustin referred to by the Examiner concerns the normalization, i.e., calibration, to account for the errors that occur as a result of significant variations in the amount of mRNA target transcripts in the starting material in RT-PCR reactions for quantifying mRNA transcripts again, particularly when comparing amplification of transcripts from samples taken from different individuals. According to Bustin, a common method for minimizingthese quantification errors between samples is to include a cellular RNA in the reaction, one that is expressed at a constant level in various tissues and, if possible, expressed at the same level endogenously as the mRNA gene transcript under study. Therefore, Bustin relates to normalization of an RT-PCR reaction using an RNA with known *in vivo* expression levels.

It is submitted that Bustin provides a method for determining how badly a multiplex amplification has gone by including an internal standard as a target. No provision for improving the results of a multiplex amplification is provided, and no teaching that can be combined with the Backus reference suggests the improvement claimed by Applicants.

As discussed above, Applicants have demonstrated that their improved method is suitable for obtaining uniform amplifications in a multiplex PCR reaction, with multiple target nucleic acids, requiring multiple primer sets. Bustin does not disclose any means for leveling the output of a multiplex PCR.

In fact, Bustin indicates that, at the time the review was published (2000), Applicants' improved results were unknown. According to Bustin,

> "**Multiplex RT-PCR** . . .
> The final difficulty [with multiplex RT-PCR] is associated with limitations caused by mutual interference of multiple sets of PCR primers, which can reduce the dynamic range of the sensitivity and make quantification unreliable. Furthermore, the efficiency of multiplex detection is significantly affected by the extension time and the concentrations of dNTPs, primers and $MgCl_2$ . . . Vastly different levels of target mRNAs will cause quantification problems even for real-time detection, as the exponential phase of amplification of the less abundant mRNA

> will not overlap with that of the highly abundant target." (See, Bustin, page 185, right column, 3rd paragraph.)

Finally, Bustin concludes,

> "Therefore, in practice, if accurate quantification is the main aim, it is probably best to limit multiplexing to the detection of two or three transcripts." (See, Bustin, page 185, right column, 3rd paragraph.)

Thus, Bustin acknowledges the prevalence of the same problems in the art that are now addressed and overcome with Applicants' method. While the Bustin reference may identify major problems existing in the field of multiplex RT-PCR, the best solution that is suggested for the problem by Bustin is reducing the number of target nucleic acids in the reaction mixture. Bustin does not teach or suggest any aspect of Applicants' method for the coamplification of two or more target nucleic acids by employing a hotstart DNA polymerase in combination with a volume exclusion agent, nor does Bustin suggest that such a reaction may be carried out without the need for primer optimization.

Birch et al. teach modification of a thermostable enzyme with a dicarboxylic acid anhydride and its use in a standard PCR reaction, i.e., to amplify a single target nucleic acid. However, Birch et al. do not teach or suggest the use of a hotstart enzyme in combination with a volume exclusion agent for use in a multiplex PCR reaction.
As such, the Birch et al. reference in combination with Backus and/or Bustin does not make up for the lack of this teaching or suggestion in either of those references.

In view of the foregoing remarks, the combination of Backus with Bustin and Birch et al. does not suggest the improvement of the present invention. Indeed, the improvement of the present invention overcomes problems that are acknowledged and illustrated by those publications. Accordingly, reconsideration and allowance of Claims 1, 2, 4-11, 16, 23, and 24 are respectfully solicited.

35 U.S.C. §103

The Examiner has rejected Claims 12-15 under 35 U.S.C. §103 as being unpatentable over Backus *supra*, in view of Bustin *supra*, and Birch et al. *supra*, and further in view of Reed et al., U.S. Pat. No. 5,459,038 ("Reed et al.") and Demeke et al., *Biotechniques*, 12(3): 333-334 (1992).

The Backus, Bustin, and Birch et al. references are as cited above. With respect to Reed et al. and Demeke et al., the Examiner states,

> "[I]t would have been prima facie obvious in view of the teachings of Demke [sic] and Reed to include dextran into the method of amplification taught by Backus in view of Bustin." (See, Office Action, page 14.)

The Demeke et al. reference simply reports that dextran did not inhibit PCR amplification of spinach DNA. (See, Demeke et al., "Results And Discussion")

And Reed et al. simply discloses a well known fact in the art of nucleic acid hybridization reactions, i.e., that inclusion of dextran in a PCR reaction increases the efficiency of amplification because, as is also disclosed in the present specification on page 6, lines 1-9,

> "It is known that the hybridization rate of nucleic acids is increased considerably in the presence of volume exclusion agents such as dextran sulfate or polyethylene glycol due to exclusion of nucleic acids from the volume of solution occupied by the agents."

Therefore, Demeke et al. and Reed et al., alone or in combination with Backus and/or Bustin, and/or Birch et al. do not teach or disclose Applicants' method of multiplex PCR amplification in the presence of a chemically-modified hotstart DNA polymerase and a volume exclusion agent.

Reconsideration and allowance of Claims 12-15 are respectfully requested.

35 U.S.C. §103

The Examiner has rejected Claim 25 under 35 U.S.C. §103 as being unpatentable over Backus *supra*, in view of Bustin *supra*, and Birch et al. *supra*, and further in view of Ivanov et al., U.S. Pat. No. 6,183,998 ("Ivanov et al.").

The Backus, Bustin, and Birch et al. references are as cited above. With respect to Ivanov et al. the Examiner states,

> "It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to have extended the teaching of Bustin and Backus to include the chemical modification through reaction with an aldehyde as taught by Ivanov." (See, Office Action, page 15.)

Ivanov et al. show the use of a hotstart specific DNA-polymerase together with a non-ionic, polymeric volume exclusion agent. However, Ivanov et al. relates to PCR reactions in general and not specifically to multiplex PCR methods, and does not contemplate or disclose the particular improvement of the present invention which relates to a means for obtaining uniform multiplex PCR results while avoiding such techniques as optimizing the primer concentrations of all primers as necessary for multiplex PCR. A person skilled in the art would thus not take from Ivanov et al. any solution for the many problems associated with multiplex PCR.

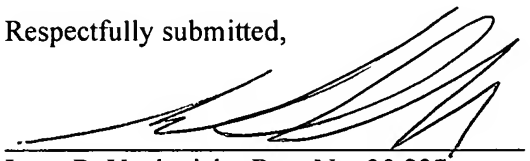
Accordingly, the combination of Ivanov et al. with Backus, Bustin or Birch et al. does not teach or suggest Applicants' method for multiplex PCR amplification.

Reconsideration and allowance of Claim 25 are respectfully requested.

For the reasons set forth above, none of the cited references, either alone or in combination, teach or suggest Applicants' improved method for the coamplification of two or more target nucleic acids.

Reconsideration and allowance of Claims 1, 2, 4-16, and 23-25, are respectfully requested.

Respectfully submitted,



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